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Phosphorylation of *Bacillus subtilis* Transcription Factor Spo0A Stimulates Transcription from the *spoIIG* Promoter by Enhancing Binding to Weak 0A Boxes

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Activation of the *spoIIG* promoter at the onset of sporulation in *Bacillus subtilis* requires the regulatory protein, Spo0A, which binds to two sites in the promoter, sites 1 and 2. Phosphorylation of Spo0A is essential for the initiation of sporulation. Therefore, we examined the role of Spo0A phosphorylation in *spoIIG* promoter activation. Phosphorylation of Spo0A stimulated transcription from the *spoIIG* promoter in vitro. In DNAse I footprinting experiments with the *spoIIG* promoter, we found that phosphorylation of Spo0A increased its affinity for site 2 more than for site 1, which is the site to which nonphosphorylated Spo0A binds most avidly. This result could not be explained by increased cooperativity between Spo0A bound at sites 1 and 2 because the increased affinity for site 2 by phosphorylated Spo0A was also observed with a deletion derivative of the *spoIIG* promoter containing only site 2. We have located Spo0A-binding sequences in the *spoIIG* promoter by DMS protection assays and mutational analysis, and found that site 1 contains one higher-affinity binding sequence whereas site 2 contains two weaker-binding sites. Two substitutions in site 2 of the *spoIIG* promoter that change the sequence to be more like an optimal Spo0A-binding site were found to increase promoter activity. Moreover, phosphorylation of Spo0A was not required in vivo for activation of the *spoIIG* promoter containing these strong binding sites. The results suggest that the primary role for phosphorylation of Spo0A is to increase its affinity for specific sites rather than to activate an activity of Spo0A that acts on RNA polymerase at promoters.

During endospore formation in *Bacillus subtilis*, transcription of sporulation-specific genes is regulated both temporally and spatially. Particularly at later times, this regulation is achieved largely by the sequential and, in some cases, compartment-specific appearance or activation of a series of secondary sigma factors that direct core RNA polymerase to different sets of promoters (reviewed in reference 6). However, at early times in the sporulation gene activation program, the regulatory protein Spo0A plays a crucial role as both a negative and a positive effector of gene expression independent of the sigma substitution cascade. As a negative effector, Spo0A controls the expression of the *abrB* gene (21), which is itself a repressor of several genes that normally become active during the transition into stationary phase. As a positive effector, Spo0A is responsible for activating the expression of at least three important stage II genes or operons, including *spoIIA* (24), *spoIIE* (28), and *spoIIG* (18), in the first 60 to 90 min of sporulation. The Spo0A protein apparently mediates both its positive and negative regulatory effects by binding to sites in the vicinity of target promoters conforming to the consensus 5′-TGNCGAA-3′, a sequence referred to as the 0A box (21).

Spo0A belongs to a superfamily of phosphorylation-activated signal transduction proteins usually referred to as response regulators, which mediate adaptive responses to environmental or metabolic signals (reviewed in references 15 and 16). Like Spo0A, many other response regulator proteins are transcription factors which are activated by phosphorylation to stimulate or repress the expression of certain target genes or operons. Response regulators generally acquire their phosphoryl group directly from a cognate histidine kinase partner, although phosphate-carrier proteins sometimes function as intermediaries between histidine kinases and response regulators (3), and noncognate kinases or low-molecular-weight phosphodonors, such as acetyl phosphate, may substitute for histidine kinases both in vitro and in vivo (13); thus the phosphorylation state of a response regulator protein rather than the agent of phosphorylation is the key determinant of function.

Multiple kinds of environmental or metabolic signals control the initiation of sporulation, and several lines of evidence indicate that these signals act mainly or exclusively by influencing the phosphorylation state of Spo0A (10). For example, mutations in Spo0A that prevent phosphorylation, such as a D56Q substitution (a substitution of glutamine for the aspartyl residue at position 56 of Spo0A), also completely abolish sporulation (8, 23). Moreover, phosphorylation of Spo0A in vitro increases its ability to stimulate transcription in vitro from the *spoIIG* promoter (2), a promoter which is activated at the onset of sporulation. Phosphorylation of Spo0A in vitro has been shown to increase its affinity for binding to at least one promoter, that of the *abrB* gene (24), at which Spo0A acts as a repressor. In the case of promoters that are positively regulated by Spo0A, such as the one controlling the expression of the *spoIIG* operon, the question arises whether phosphorylation might similarly mediate a regulatory effect simply by enhancing binding affinity. The *spo0A* gene is also known to be autogenously regulated at the level of transcription (23, 26), raising the possibility that the Spo0A protein may accumulate to significantly higher levels at the onset of sporulation. In previous work (18, 19), it was shown that unphosphorylated Spo0A protein binds to two regions of the *spoIIG* promoter, sites 1 and 2. If the amounts of Spo0A protein increase significantly after the cessation of growth, the possibility arises that stimulation of *spoIIG* promoter activity at the onset of

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sporulation might result largely from the increased accumulation of Spo0A at that time.

In the present work, we investigated the role of Spo0A phosphorylation in *spoIIG* promoter activation in vitro and in vivo. We found that phosphorylation of Spo0A increased its binding affinity for the *spoIIG* promoter, especially for site 2. We found also that site 2 actually consists of two weak Spo0A-binding sites. Our results suggest that phosphorylation of Spo0A may stimulate expression from promoters such as that of *spoIIG* by enhancing its affinity for weak 0A box-binding sites. We confirmed by Western blot (immunoblot) analysis with anti-Spo0A antibodies that the levels of Spo0A protein do increase significantly at the onset of sporulation and that this increase alone, independent of phosphorylation, might mediate some regulatory interactions between Spo0A and target promoters possessing strong binding sites.

(Some of these results, including the effects of Spo0A phosphorylation on its ability to stimulate *spoIIG* transcription in vitro and on its binding to the *spoIIG* promoter, were presented at the Eleventh International Spores Conference, Woods Hole, Mass., May 1992.)

MATERIALS AND METHODS

Mutagenesis of the *spoIIG* promoter and β-galactosidase assays. The −45G and −38C/−45G promoters were made by oligonucleotide-directed mutagenesis and introduced into *B. subtilis* JH642 as described by Satola et al. (18).

Partial purification of Spo0A and in vitro phosphorylation. Spo0A was partially purified from strain EUS9011 (*Escherichia coli* JM107 containing pKK233-3) as described by Satola et al. (18), with the following exceptions. After the protein was fractionated by heparin agarose chromatography, the peak fraction containing Spo0A was equilibrated in a buffer of 300 mM KCl, 20 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 1 mM EDTA, 50 μg of phenylmethylsulfonyl fluoride (PMSF), and 3% glycerol and fractionated by gel filtration chromatography on either a 180-ml Sephadex G-75 or 120-ml Superdex 200 column. Fractions were analyzed by Coomassie blue staining after sodium dodecyl sulfate-(SDS) polyacrylamide gel electrophoresis, and those fractions containing Spo0A were stored as previously described (18). These fractions appeared to contain more than 95% Spo0A.

Spo0A was phosphorylated in vitro by incubation with purified NR_{II} and ATP. To quantitate the amount of phosphorylation, 11 μM Spo0A was incubated with 0.8 μM NR_{II} and 1.7 mM [γ-^{32}P]ATP (specific activity of 2.35 Ci/mmol) in the same buffer described in the footprint procedure at 37°C for 10 min. Labeled NR_{II} and Spo0A were separated by SDS-polyacrylamide gel electrophoresis. The Spo0A band was extracted, and the radioactivity was counted in a scintillation counter. Approximately 1% of Spo0A was phosphorylated by this procedure. Since only a small fraction of the Spo0A was phosphorylated by NR_{II}, we also used the low-molecular-weight phosphodonor acetyl phosphate, which has been used to specifically phosphorylate several response regulators in autophosphorylation reactions (13). Spo0A was incubated with 50 mM acetyl phosphate in the footprint buffer. The extent of autophosphorylation was not determined directly. However, acetyl phosphate-treated Spo0A worked as efficiently as NR_{II} phosphorylated Spo0A in footprint protection assays. We also found that pretreatment of Spo0A with 50 mM phosphoenolpyruvate (PEP) increased the affinity of Spo0A for its binding sites in footprinting assays. From these results it seems likely that PEP also acts as a phosphodonor in Spo0A autophosphorylation reactions.

In vitro transcription assays. Isolation of the RNA polymerase containing σ^A used in the in vitro transcription assays was described previously by Satola et al. (19). Briefly, polymerase was isolated from *B. subtilis* ML1 by the phase-partitioning procedure, followed by chromatography on Sephacryl 300 and DNA cellulose.

The transcription reactions were done essentially as described previously (19), except the reaction mixture volume was 20 μl in a buffer that consisted of 33 mM Tris acetate (pH 7.9), 10 mM Mg^{2+} Acetate, 0.5 mM dithiothreitol, 0.15 mg of bovine serum albumin per ml, (BSA), and 66 mM K^+ acetate. Spo0A that had been phosphorylated by NR_{II} was diluted 10-fold in the transcription buffer (final concentration of 1.1 μM Spo0A) and incubated at 37°C for 10 min with 0.025 μM linearized DNA and approximately 0.03 μM σ^A-RNA polymerase ($E\sigma^A$). The ribonucleoside triphosphates (1 μl of ATP, UTP, and GTP at 1.5 mg/ml [each]) and 10 μCi of [α-^{32}P]CTP (800 Ci/mmol) were added to the reaction mixture and incubated for 30 s before 12 μg of heparin was added. The linear DNA templates used were described previously (19).

DNase I protection assays. DNase I protection assays were done in a reaction mixture volume of 50 μl in a buffer consisting of 52 mM Tris acetate (pH 7.5), 70 mM K^+ acetate, 8% glycerol, 1.1 mM EDTA, 0.7 mM dithiothreitol, 7 mM $MgCl_2$, 3 mM $CaCl_2$, and 0.05 mg of BSA per ml. The Spo0A protein was phosphorylated as described above and incubated with approximately 1 nM DNA at 37°C for 10 min. The digestion and precipitation of the reaction mixture were done essentially as described by Satola et al. (18).

The wild-type *spoIIG* promoter DNA cloned into pUCII-GtrpA (19) and Δ78 cloned into pJH101 (7) were labeled at the *Bam*HI site so that the nontranscribed strand was labeled. Second digestions were done at *Eco*RI for pJH101 and *Pvu*II for pUCIIGtrpA. The *spoIIA* promoter DNA on pPP157 (25) was labeled at the *Ava*I site and digested again with *Ssp*I so that the nontranscribed strand was labeled.

DMS methylation protection assays. Spo0A was incubated with DNA in the same buffer as that used in the DNase I protection assays at 37°C for 10 min. Immediately before use, DMS was diluted to 150 mM in water, and 5 μl of diluted dimethylsulfate (DMS) was incubated with the DNA and protein for 5 min at 37°C. The reaction was quenched, and the reaction mixture was treated with piperidine as described by Sasse-Dwight and Gralla (17), except that after piperidine treatment the reaction mixtures were dried under vacuum, resuspended in 50 μl H_2O, and ethanol precipitated. The precipitated DNA was resuspended in 50 μl H_2O and used in a modified PCR reaction (17) with a single primer labeled by [γ-^{32}P]ATP and T4 kinase. The DNA was supercoiled plasmid DNA: pUCIIGtrpA for the *spoIIG* promoter and pPP157 for the *spoIIA* promoter.

Cloning of 0A box in pUC19. Complementary oligonucleotides with the sequence 5′-AGCTGCAGCTGTCGAACTAG-3′ and 5′-AGCTCTAGTTCGACAGCTGC-3′ that contained a potential 0A box were synthesized, annealed, and cloned into the *Hin*dIII site of pUC19 (27). They were 3′-end labeled at the *Eco*RI site and digested again with *Bgl*I.

Western analysis of Spo0A protein. Polyclonal anti-Spo0A antibodies were raised in rabbits by using heparin agarose-purified Spo0A protein as an antigen. Crude extracts of *B. subtilis* strains were prepared from cells grown in DSM at 37°C. Samples (10 to 25 ml) were collected at selected times and washed in a buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 10% glycerol, 1 M KCl, and 1.7 mM PMSF. Cell pellets were resuspended in a buffer containing 10 mM Tris-HCl (pH 8.4), 1 mM EDTA, 10 mM $MgCl_2$, 0.3 mM dithio-

threitol, and 1.7 mM PMSF and stored at −70°C. Cells were broken by sonication (three times for 10 s each at 50 W). Protein samples (50 μg) were subjected to electrophoresis through an SDS–12% polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) by electrophoresis, and Spo0A protein was detected with the Promega Protoblot Western Blot Alkaline Phosphatase System, as described by the manufacturer.

Membranes were blocked by incubation at room temperature in Tris-buffered saline (TBS) containing 0.05% Tween (TBST) plus 1% BSA for 30 min. To bind Spo0A antibody, the blocking solution was replaced with TBST containing a 1:5,000 dilution of rabbit anti-Spo0A antiserum (20 ml of TBST plus 4 μl of antiserum) and the mixture was incubated with gentle agitation at room temperature for 60 min. To remove unbound anti-Spo0A antibodies, the membrane was washed three times in TBST for 5 to 10 min each. The membrane was then transferred to TBST containing a 1:5,000 dilution of anti-rabbit immunoglobulin G-alkaline phosphatase conjugate and incubated for 30 min with gentle agitation. The membrane was washed three times in TBST for 5 to 10 minutes each to remove unbound anti-rabbit immunoglobulin G. The membrane was rinsed briefly in two changes of TBS to remove the Tween. To detect protein bands, the membrane was transferred to 10 ml of alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, and 5 mM $MgCl_2$) containing 66 μl of Nitro Blue Tetrazolium color development substrate and 33 μl of BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium) color development substrate and incubated for 1 to 10 min with gentle agitation. The color reaction was stopped by washing the membrane for several minutes in deionized water.

RESULTS

Phosphorylation of Spo0A stimulates transcription from the *spoIIG* promoter in vitro. Burbulys et al. (3) have shown that Spo0A can be phosphorylated in vitro by the *B. subtilis* phosphorelay proteins in which the phosphoryl group acquired by autophosphorylation of the histidine kinase, KinA, is transferred to Spo0F, then to Spo0B, and finally to Spo0A. However, like other response regulators, Spo0A can also acquire a phosphoryl group in vitro from heterologous histidine kinases, such as CheA, EnvZ, and NR_{II} (1, 14). Also, seven other response regulator proteins have been found to autophosphorylate efficiently and specifically in the presence of low-molecular-weight phosphodonors (13). In order to examine the effects of Spo0A phosphorylation on its interactions with the *spoIIG* promoter, we phosphorylated Spo0A in vitro with either the histidine kinase NR_{II} or low-molecular-weight phosphodonors. In all of our experiments the phosphorylation of Spo0A was inefficient (about 1% of the Spo0A was phosphorylated by NR_{II} [see Materials and Methods]). We do not know whether most of the Spo0A in our preparations was inactive and therefore could not be phosphorylated or whether the conditions for phosphorylation were inefficient. It is unlikely that Spo0A, which was purified from *E. coli*, was already phosphorylated. If a significant fraction of the Spo0A were already phosphorylated, then phosphorylation of an additional 1% would not be expected to have significant effects on binding and transcription (described below). We first tested whether phosphorylation of Spo0A affected its ability to stimulate transcription from the *spoIIG* promoter.

Spo0A that had been phosphorylated by incubation with NR_{II} and ATP was added to an in vitro transcription runoff assay with *spoIIG* promoter template and σ^A-associated RNA polymerase from *B. subtilis*. This phosphorylated Spo0A stimulated transcription from the *spoIIG* promoter more efficiently than did the untreated (nonphosphorylated) Spo0A (Fig. 1). This stimulation was specific for the *spoIIG* promoter since phosphorylated Spo0A did not activate transcription from the Spo0A-independent promoter *tms*.

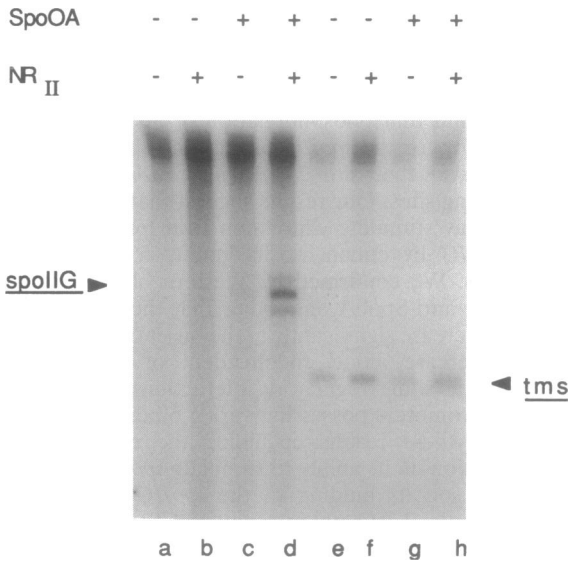

FIG. 1. Phosphorylated Spo0A stimulates transcription from the *spoIIG* promoter in vitro. We assayed the effects of Spo0A or Spo0A that had been phosphorylated on in vitro transcription reactions by using linear DNA templates containing the *spoIIG* promoter (lanes a to d) or the *tms* promoter (lanes e to h). Each reaction mixture contained 0.025 μM linear DNA template and approximately 0.03 μM $E\sigma^A$. Spo0A (11 μM) was phosphorylated by incubation with 0.8 μM NR_{II} and 1.7 mM ATP at 37°C for 10 min and then diluted to 1 μM Spo0A in the transcription reaction mixtures (lanes a to h). Spo0A or NR_{II} was omitted from some reaction mixtures as indicated above the lanes. The transcripts from the *spoIIG* and *tms* promoters are indicated by the arrowheads. Samples were subjected to electrophoresis in denaturing polyacrylamide gels followed by autoradiography. *Hpa*II-cleaved pBR322 fragments were used as molecular size markers (data not shown).

Phosphorylation of Spo0A increases its affinity for the *abrB* promoter (24). Therefore, we tested whether phosphorylation of Spo0A affects its binding to the *spoIIG* promoter. In DNase I footprint assays, Spo0A that was not phosphorylated with NR_{II} partially protected site 1. In contrast, the same amount of Spo0A that had been phosphorylated protected both site 1 and site 2 (Fig. 2). Therefore, phosphorylated Spo0A has a higher affinity for the *spoIIG* promoter than nonphosphorylated Spo0A. Since it was not known what fraction of Spo0A was active in each preparation, it is not possible to make quantitative estimates of the relative activities of Spo0A and phosphorylated Spo0A in the transcription and binding assays. However, in every case the phosphorylated form of Spo0A was more active in these assays than the nonphosphorylated Spo0A.

We also tested whether Spo0A bound to the promoter would stabilize the interaction of $E\sigma^A$ with the promoter in DNase I protection assays. Addition of $E\sigma^A$ and ATP had little effect on the pattern of DNAse cleavage in the footprint assay (Fig. 2, lane e). However, when we added both $E\sigma^A$ and phosphorylated Spo0A, the region protected from the DNAse included both site 1 and site 2 and there was an extension of the footprint through the −10 region of the promoter (Fig. 2,

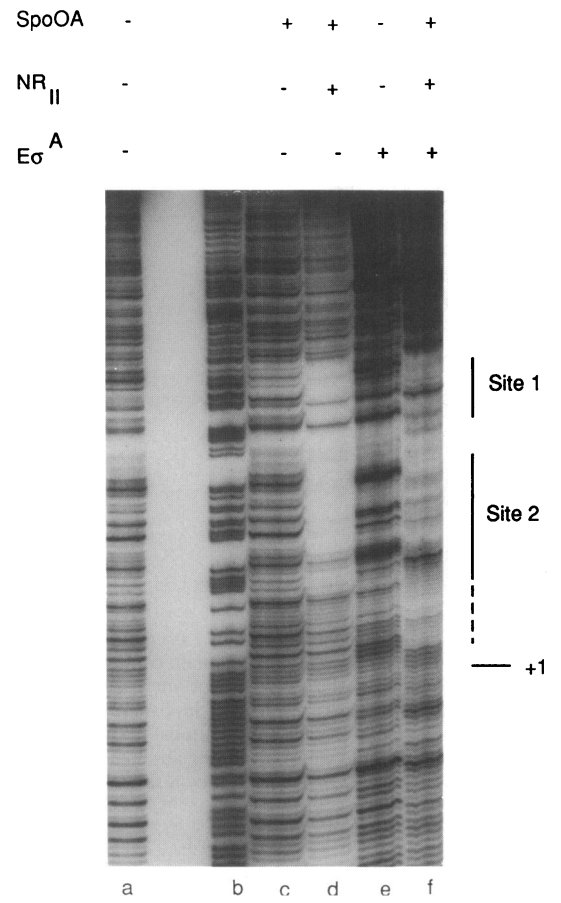

FIG. 2. DNase I footprint assays of Spo0A, phosphorylated Spo0A, and $E\sigma^A$ binding to *spoIIG* promoter. The DNA template containing the *spoIIG* promoter was radiolabeled on the nontranscribed strand and digested with DNase I in the presence or absence of Spo0A, $E\sigma^A$, or Spo0A that had been phosphorylated by preincubation with NR_{II} and ATP as indicated above each lane (lanes a and c to f). Spo0A (1.9 μM) was preincubated with 0.54 μM NR_{II} at 37°C for 10 min and then 1 nM of end-labeled DNA; in the reaction mixtures indicated, approximately 48 pM $E\sigma^A$ was added, and the mixtures were incubated at 37°C for an additional 10 min. All reaction mixtures contained 0.4 mM ATP. Lane b contains the products of the same DNA template that had been cleaved at the adenosine and guanosine residues by the chemical sequencing reactions. The regions corresponding to sites 1 and 2 are indicated by a solid line, and the extension of the footprint due to $E\sigma^A$ is indicated by a dashed line.

lane f). This additional protection probably results from $E\sigma^A$ binding to the promoter. We also noted that when both RNA polymerase and phosphorylated Spo0A were bound, the DNase I protection patterns for sites 1 and 2 were slightly different from those produced when only phosphorylated Spo0A was bound. This is not surprising since bound RNA polymerase would be expected to closely contact these sites, and its close proximity to these sites might be expected to alter the cleavage patterns in these regions. From these results it appears that Spo0A stabilizes the interaction of $E\sigma^A$ with the promoter. However, we note that the footprint does not extend past the start point of transcription. This type of footprint is reminiscent of closed complexes characterized by Cowing et al. (5) and Schickor et al. (20). These types of complexes may be different from those formed by *B. subtilis* RNA polymerase; however, this observation raises the possibility that the majority of the RNA polymerase-promoter complexes, which produced the footprint, may not represent fully activated transcription complexes. Therefore, these results do not eliminate the possibility that Spo0A also stimulates the rate of an additional step in promoter utilization subsequent to the initial binding of RNA polymerase.

Phosphorylation of Spo0A differentially affects binding to sites 1 and 2. To determine whether phosphorylation of Spo0A enhanced binding to site 1 and site 2 equally, we examined the DNase I footprint pattern by using a range of different Spo0A concentrations (Fig. 3). The amount of protection of site 1 with phosphorylated Spo0A and nonphosphorylated protein was similar at each concentration tested. However, site 2 was protected more efficiently by 6.0 μM Spo0A that had been phosphorylated than by 6.0 μM untreated Spo0A (Fig. 3, contrast lanes e and k). Evidently, phosphorylation of Spo0A increases its affinity for site 2 but does not appear to have as much effect on its affinity for site 1.

We considered two hypotheses that would explain the effect of Spo0A phosphorylation on binding to site 2. We have previously reported that site 1 is a higher-affinity binding site for nonphosphorylated Spo0A, and that binding of nonphosphorylated Spo0A to this site facilitates binding to site 2. Thus, phosphorylation might increase cooperative binding so that once site 1 is occupied, site 2 is more readily bound by Spo0A. Another possibility is that phosphorylated Spo0A has a higher affinity for site 2 and that this effect is independent of site 1. We tested these hypotheses by footprint assays with a *spoIIG* promoter from which site 1 had been deleted. Figure 4 shows that in DNase footprinting experiments phosphorylation of Spo0A resulted in increased protection of site 2 on a DNA fragment that did not contain site 1. We concluded that phosphorylation of Spo0A can increase its affinity for site 2 independently of site 1. However, we also noted that site 2 appeared protected less efficiently when site 1 was deleted; so, the weak cooperative interactions between Spo0A bound at sites 1 and 2 described previously (19) may contribute to the overall degree of Spo0A binding to the wild-type *spoIIG* promoter.

The sequences in the *spoIIG* promoter that signal binding of Spo0A. Previous results of a mutational analysis of the *spoIIG* promoter showed that the region of site 2 needed for promoter activity is larger than a single 0A box (18). This observation raises the possibility that additional sequences are required to signal Spo0A binding or some of these sequences signal binding of another factor required for *spoIIG* promoter activity. In order to define more precisely the sequences that signal Spo0A binding, we looked at the ability of Spo0A to protect the promoter from DMS methylation. Strauch et al. (21) showed that Spo0A protects two G residues in the putative Spo0A-binding site (the 0A box), TGNCGAA, of the *abrB* promoter from DMS methylation: the G in the second position and the G complementary to the C in the fourth position. If Spo0A binds to the *spoIIG* promoter in the same manner, then the results of DMS protection assays may indicate important Spo0A-binding sequences. We found that two G residues were protected on the nontranscribed strand of the *spoIIG* promoter at position −90 (site 1) and −37 (site 2). There was also a hypermethylated base at position −20. The transcribed strand had three G residues protected at position −88 (site 2), −45 (site 2), and −35 (site 2) (Fig. 5). All of these bases were found in sequences that share some homology to the 0A box, but these sequences are in the reverse orientation (Fig. 6) from the 0A boxes in the *abrB* promoter.

The *spoIIA* promoter contains multiple 0A boxes. Potential 0A boxes have been identified in other Spo0A-dependent

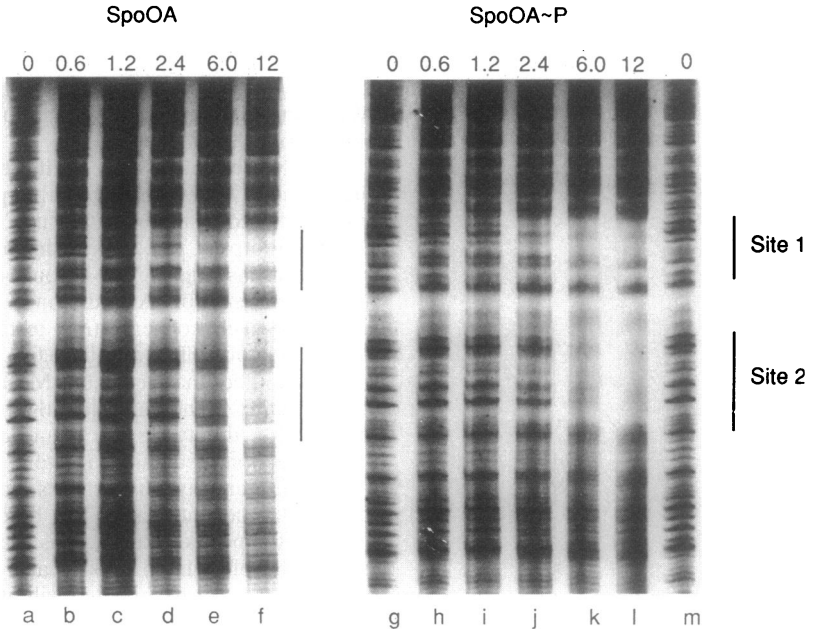

FIG. 3. Titration of Spo0A and phosphorylated Spo0A in DNase I footprint assays with *spoIIG* promoter. DNA template containing the *spoIIG* promoter was radiolabeled as described in the legend to Fig. 2 and digested with DNase I in the presence of increasing amounts of nonphosphorylated (lanes b to f) or phosphorylated (lanes h to l) Spo0A. For the reaction mixtures with phosphorylated Spo0A, 12 μM Spo0A was preincubated with 0.8 μM NR_{II} and 1.7 mM ATP for 10 min at 37°C and diluted in footprint buffer (see Materials and Methods) to the concentration (micromolar) of Spo0A indicated above each lane. Nonphosphorylated Spo0A indicates protein incubated with NR_{II} in the absence of ATP. Spo0A was omitted from the reaction mixtures in lanes a, g, and m. The regions corresponding to sites 1 and 2 are indicated by a solid line.

promoters. To further characterize the sequences that signal binding of Spo0A, we examined its binding to these hypothetical Spo0A-binding sites in one other promoter, *spoIIA*. Trach et al. (24) demonstrated that phosphorylated Spo0A increased transcription from the *spoIIA* promoter in vitro. We compared the ability of phosphorylated Spo0A and nonphosphorylated Spo0A to bind to this promoter by titrating the protein in DNase I footprint assays. Again we found that phosphorylated protein had an increased affinity for the promoter. A region extending from approximately −87 to −33 was protected by the phosphorylated protein. An additional region from −28 to −13 was also protected at the highest concentration of phosphorylated protein (Fig. 7).

We also used DMS protection assays to characterize Spo0A binding to the *spoIIA* promoter. Our results revealed several protected bases on both strands of the promoter. On the nontranscribed strand there were five G residues protected, at positions −72, −64, −49, −44, and −21. The transcribed strand was protected at positions −62, −52, −42, and −19. There was also a hypermethylated band at position −40 (Fig. 8). Some of the protected bases were in three potential 0A boxes as indicated in Fig. 9. However, three protected bases, −72, −52 and −49, did not appear to be in sequences that resemble an 0A box. There is an additional potential 0A box located at positions −87 to −81 (Fig. 9). This box is in the reverse orientation from the others in the promoter. We found no evidence that Spo0A protects the DNA within this box from methylation. It appears that in the *spoIIA* promoter the 0A boxes signal binding of Spo0A and may also facilitate binding to adjacent non-0A-box-like sequences by cooperative interactions.

A Spo0A box is sufficient to signal binding. Interpretation of the binding studies of Spo0A with the *spoIIG* and *spoIIA* promoters may be complicated by the presence of multiple

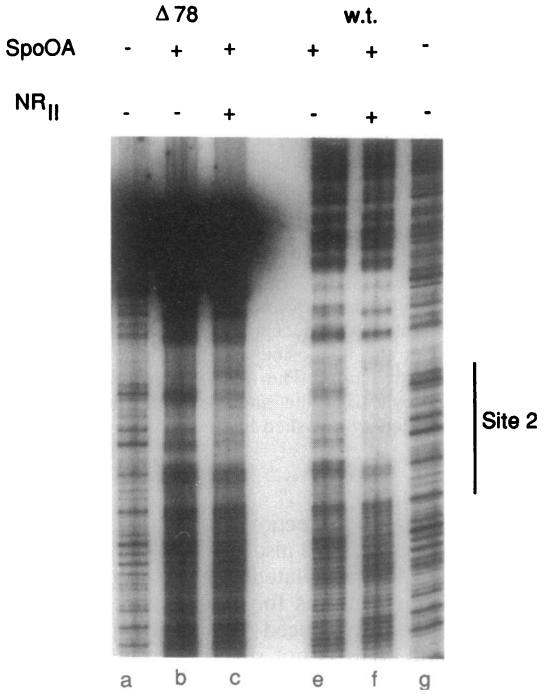

FIG. 4. DNase I footprinting assays of Spo0A and phosphorylated Spo0A binding on *spoIIG* promoter lacking site 1. The Δ78 *spoIIG* promoter has a 13-bp deletion from positions −90 to −78 so that most of site 1 has been deleted. The DNA templates were radiolabeled on the nontranscribed strand and digested in the presence of 11 μM Spo0A that was phosphorylated in the same way as that described in the legend to Fig. 3 (lanes c and f) or 11 μM nonphosphorylated Spo0A in which NR_{II} was omitted from the reaction mixture (lanes b and e). Spo0A was omitted from the reaction mixtures in lanes a and g. The solid lines indicates the region corresponding to site 2.

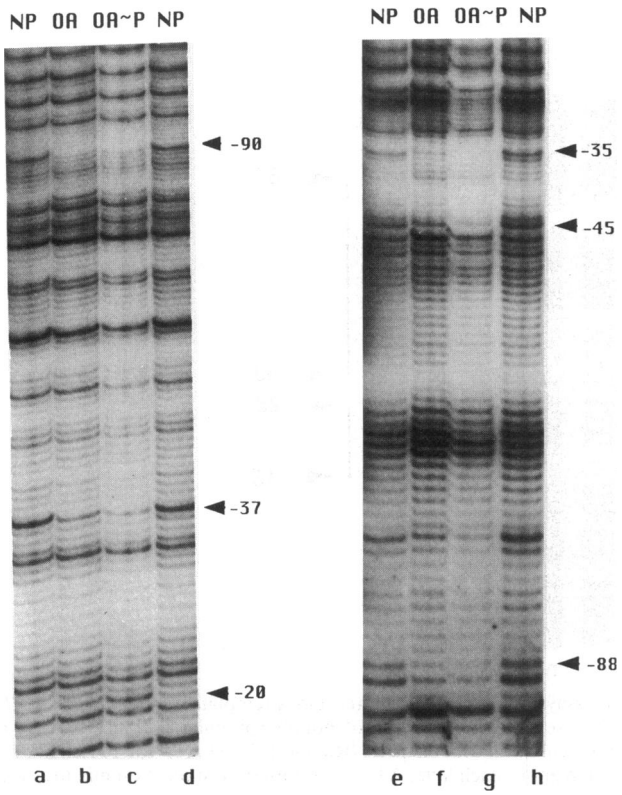

FIG. 5. DMS methylation protection assays of Spo0A and phosphorylated Spo0A binding on *spoIIG* promoter. Supercoiled DNA template (0.23 nM) containing the *spoIIG* promoter was treated with DMS after incubation with phosphorylated (lanes c and g) or nonphosphorylated (lanes b and f) Spo0A for 10 min at 37°C. Spo0A (9.4 μM) was phosphorylated by preincubation with 50 mM PEP at 37°C for 10 min (see Materials and Methods). In reaction mixtures with nonphosphorylated Spo0A, PEP was omitted. Spo0A was omitted from the reaction mixtures in lanes a, d, e, and h. DNA treated with DMS was cleaved with piperidine and used as template DNA in a modified PCR reaction with a single radiolabeled primer (see Materials and Methods). The PCR products were subjected to electrophoresis on a denaturing polyacrylamide gel followed by autoradiography. Lanes: a to d, nontranscribed strand; e to h, transcribed strand. The positions of protected or hypermethylated bases are indicated by arrowheads.

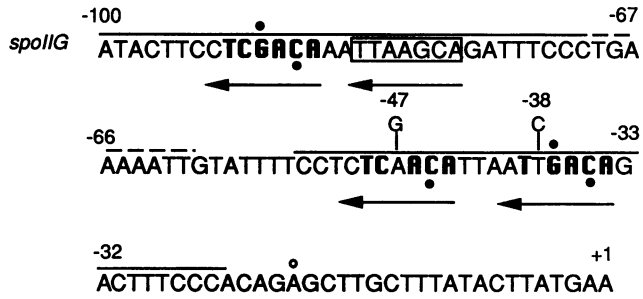

FIG. 6. Summary of DNase I and DMS protection assays with *spoIIG* promoter. Closed circles indicate protection from DMS methylation, and open circles indicate hypermethylated bases. Solid lines over these sequences indicate regions protected from DNase cleavage. Dashed lines indicate regions not cleaved by DNase. Bold letters are bases that correspond to the consensus Spo0A-binding sequence (Fig. 13). Arrows indicate the orientation of the Spo0A-binding sequence, pointing right indicates an orientation opposite to that of the usual presentation of the OA box consensus sequence, which is 5′-TGTC GAA-3′ on the nontranscribed strand. The base-pair substitutions which increase promoter activity are indicated above positions −38 and −47. The boxed sequence shares sequence homology with a Spo0A-binding site, but there is no evidence that it is important for promoter activation (see Discussion).

binding sites in these promoters and possibly by other subtle structural elements of the promoters. To test whether the 0A box consensus sequence is sufficient to signal binding of Spo0A independent of its sequence context, we introduced a potential Spo0A-binding site into the polylinker region of pUC19 and looked for binding by DNase I footprint analysis. A 20-bp insert containing the sequence 5′-TGTCGAA-3′ was sufficient to create a Spo0A-binding site that was partially protected by nonphosphorylated Spo0A and protected to a greater extent with phosphorylated Spo0A in DNase I footprinting experiments (Fig. 10). An otherwise identical insert with a single base change creating the sequence 5′-TGTCGAG-3′ and pUC19 without an insert were not protected by the same concentration of phosphorylated Spo0A (data not shown).

Testing the role of 0A boxes in site 2 of the *spoIIG* promoter. The sequence in site 1 of *spoIIG* is most similar to the 0A box consensus sequence, whereas the two sequences in site 2 are less similar to the 0A box consensus sequence, with 5 of 7 bases being identical in each. Previously, we had reported that a mutation at position −38 from a T to C, which makes the downstream 0A box in site 2 more like consensus sequences, increases the promoter's activity in vivo fourfold (18). Therefore, we predicted that if the 0A box extending from −50 to −44 were also important for *spoIIG* promoter activity, then a change at position −47 from an A to a G, which makes this sequence more like the consensus sequence, would increase the promoter activity in vivo. The mutation was made in a *spoIIG* promoter, fused to *lacZ*, and introduced into the chromosome of BG306 (wild-type *spo0A*). The change to G at position −47 (referred to hereafter as −47G) increased the promoter activity approximately fivefold compared with that of the wild-type promoter (Fig. 11). We also constructed a promoter with both the −47G and −38C mutations so that both 0A boxes in site 2 would be strong. This promoter was about 10-fold more active than the wild-type promoter (Fig. 11).

High-affinity *Spo0A*-binding sites make transcription activation independent of phosphorylation in vivo. The double-mutant *spoIIG* promoter (−38C/−47G), which contains consensus sequence-like 0A boxes at site 2, exhibited more activity in the exponential-phase culture than that seen from the wild-type promoter during sporulation. We hypothesized that this was due to either a small amount of phosphorylated Spo0A that is present during vegetative growth or nonphosphorylated Spo0A that can activate the promoter, because the mutations in site 2 create higher-affinity binding sites. Therefore, we examined the activity of the −38C/−47G promoter in strains that have a phosphorylation-deficient form of Spo0A. Green et al. (8) constructed a mutation in Spo0A that changed the conserved aspartic acid residue at position 56 to a glutamine (D56Q). The aspartic acid at position 56 is probably the site of phosphorylation, and the mutant Spo0A protein could not be phosphorylated in vitro. Moreover, this substitution in Spo0A resulted in a Spo$^-$ phenotype (8). We introduced a −38C/−47G promoter-*lacZ* fusion into the BG314(D56Q) background by transduction with SPβ phage. β-Galactosidase assays showed that the double-mutant promoter was still activated in the D56Q background with a peak activity that was

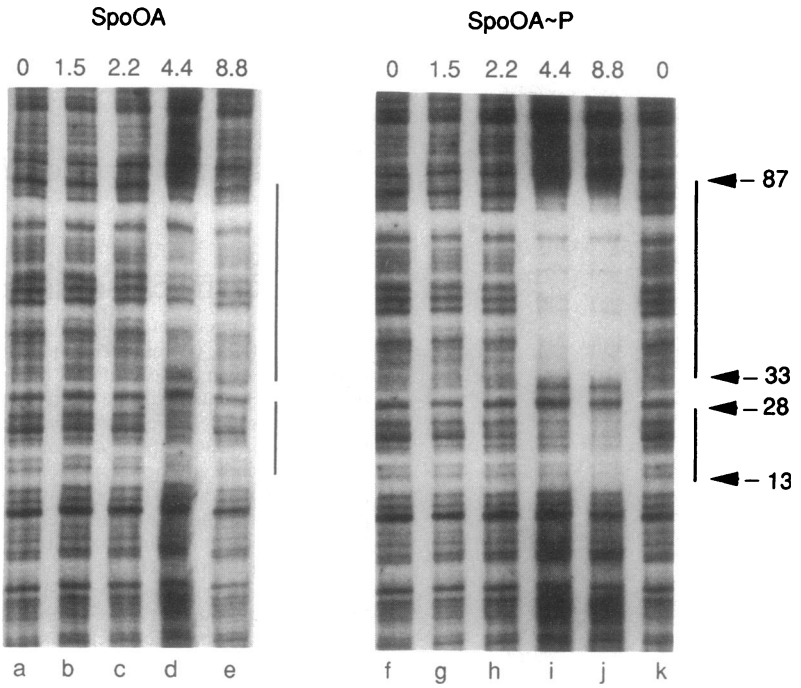

FIG. 7. Titration of Spo0A and phosphorylated Spo0A in DNase I footprint assays with *spoIIA* promoter. DNA template containing the *spoIIA* promoter was radiolabeled on the nontranscribed strand and treated with DNase I in the presence of nonphosphorylated (lanes b to e) or phosphorylated (lanes g to j) Spo0A. Spo0A (8.8 μM) was phosphorylated by preincubation with 0.8 μM NR_{II} and 1.7 mM ATP for 10 min at 37°C and then diluted with footprint buffer to the concentrations (micromolar) indicated above each lane. ATP was omitted from reaction mixtures with nonphosphorylated Spo0A, and Spo0A was omitted from the reaction mixtures in lanes a, f, and k. Regions of protection are indicated by solid lines, and the positions on the promoter are indicated by arrowheads.

about 75% of peak wild-type promoter activity in a wild-type *spo0A* background (Fig. 12). This activation was dependent on Spo0A since the −38C/−47G promoter was not active in an isogenic strain containing a null allele of *spo0A* (resulting in a deletion of the 75 amino acids from the amino terminus of Spo0A). In addition, D56Q was not able to activate the wild-type promoter. Since the addition of strong Spo0A-binding sites in the *spoIIG* promoter partially suppressed the effect of the D56 substitution, we suggest that phosphorylation at D56 is not essential for activation of transcription, but rather that phosphorylation stimulates the protein's affinity for DNA.

Quantitation of Spo0A protein in wild-type and D56Q mutant extracts. Interpreting the relative activities of the wild-type and −38C/−47G mutant *spoIIG* promoters in wild-type and D56Q mutant strains is complicated by the fact that the *spo0A* gene is apparently autogenously regulated (4, 9, 26); if Spo0A protein accumulates to higher levels in the wild type than in the mutant, we may be underestimating the ability of the D56Q mutant form of Spo0A to utilize the −38C/−47G promoter. To investigate this possibility, Spo0A protein levels in extracts of wild-type and D56Q mutant strains were visualized by Western blot analysis at different times during growth and sporulation (Fig. 13). Although Spo0A levels were roughly equal during exponential growth phase, levels increased sharply in the wild type during stationary phase, while remaining constant in the mutant. From T_2 through T_4, the wild-type extracts appeared to contain three to six times as much Spo0A protein. Relative protein levels were evaluated more carefully at T_3 by probing blots prepared with serial dilutions of both extracts. The results indicated 3.5-fold-higher levels of protein in the wild type (data not shown). We conclude that phosphorylation of Spo0A is required for its increased expression in stationary-phase cultures. Moreover, normalizing for the different levels of Spo0A protein present in wild-type and D56Q mutant extracts, we conclude that nonphosphorylated Spo0A protein can stimulate transcription from the −38C/−47G mutant *spoIIG* promoter almost as well as phosphorylated Spo0A protein stimulates the wild-type promoter.

DISCUSSION

We found previously that high concentrations of nonphosphorylated Spo0A stimulated transcription from the *spoIIG* promoter in vitro. However, genetic evidence suggests that the phosphorylated form of Spo0A is essential for *spoIIG* promoter activity in vivo (11, 12). Two models could explain these results. In one, the phosphorylation of Spo0A is required to stimulate its own synthesis from a Spo0A-dependent promoter that drives transcription of the *spo0A* gene (23, 26). The accumulation of a high level of nonphosphorylated Spo0A at the onset of sporulation would then stimulate *spoIIG* promoter activity. The second model, in which phosphorylation of Spo0A results in a form that has a higher affinity for the *spoIIG* promoter, is supported by the results presented here. We found that phosphorylation of Spo0A stimulates its ability to activate *spoIIG* transcription in vitro and that phosphorylation of Spo0A appears to increase its affinity for the *spoIIG* promoter, especially for site 2, the weaker binding site. It is not known what fraction of Spo0A is phosphorylated in vivo; however, since phosphorylation of a small fraction of Spo0A in vitro was sufficient to enhance binding to the *spoIIG* promoter, it is likely that the phosphorylated form of Spo0A activates the *spoIIG* promoter directly in vivo.

The region called site 2 in the *spoIIG* promoter was defined

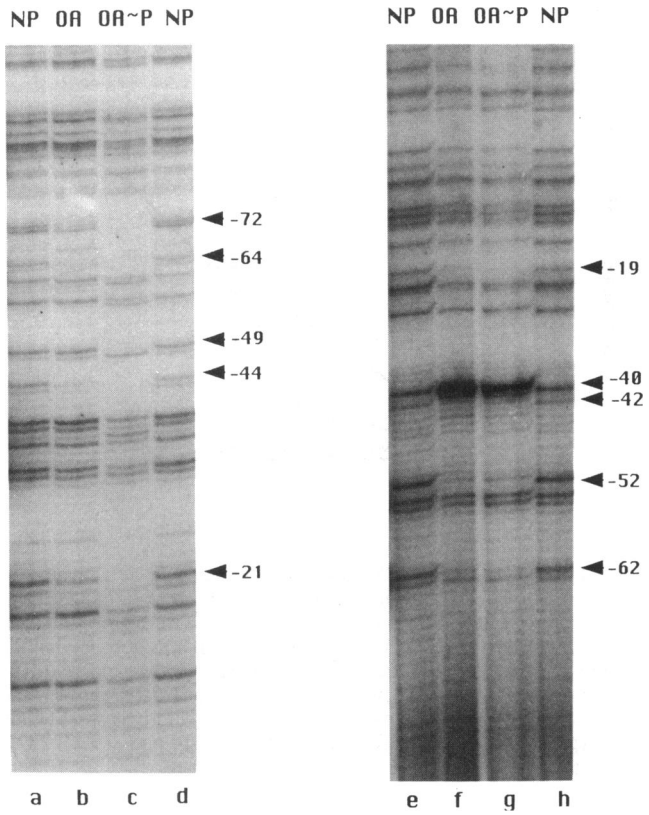

FIG. 8. DMS methylation protection assays with *spoIIA* promoter and Spo0A and phosphorylated Spo0A. The concentrations of protein and DNA are described in the legend to Fig. 5. The reaction mixtures contained no Spo0A (lanes a, d, e, and h), nonphosphorylated Spo0A (lanes b and f), or phosphorylated Spo0A (lanes c and g). Lanes: a to d, nontranscribed strand; e to h, transcribed strand. The positions of protected and hypermethylated bases are indicated by arrowheads.

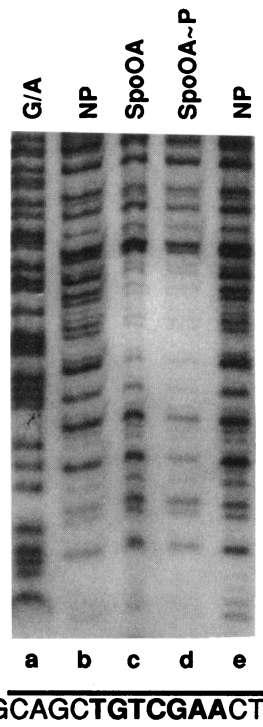

FIG. 10. DNase I footprint assays of Spo0A and phosphorylated Spo0A binding to a single 0A box in the pUC19 polylinker. DNA template was radiolabeled on the nontranscribed strand and cleaved by DNase I in the presence of 7 μM Spo0A (lane c) or the same concentration of Spo0A that had been phosphorylated by incubation with 50 mM acetyl phosphate for 10 min at 37°C (lane d). Spo0A was omitted from the reaction mixtures in lanes b and e. Lane a contains the products of the same DNA that was cleaved at the adenosine and guanosine residues by chemical sequencing reactions. The sequence shown (bottom) corresponds to the sequence cloned into the *Hin*dIII site of pUC19, and the solid line indicates those bases which were protected from DNase cleavage.

previously by point mutations that affect *spoIIG* promoter activity (18). This region is larger than a single Spo0A-binding site, and our results suggest that it is composed of two Spo0A-binding sites. In order to determine the sequences that signal Spo0A binding in the *spoIIG* promoter, we have taken three approaches. We used DMS protection assays to determine the base pairs that are closely contacted by Spo0A and mutagenesis to test the role of specific base pairs. We also characterized additional Spo0A-binding sites on another promoter, *spoIIA*.

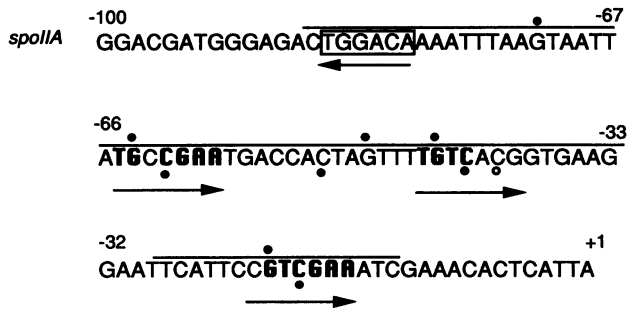

FIG. 9. Summary of DNase I and DMS protection assays with *spoIIA* promoter. The symbols are described in the legend to Fig. 6.

Spo0A protected bases from methylation in three regions of the *spoIIG* promoter that share homology to the previously published 0A box TGNCGAA. One sequence is located in site 1, and two sequences are located in site 2. We believe that these sequences are important for Spo0A binding because previously reported mutations in these sequences decreased the activity of the promoter in vivo and were found to create lower-affinity binding sites for Spo0A (18, 19). Two mutations in site 2, −47G and −38C, which make the two downstream sequences more like the sequence in site 1, significantly increase the promoter activity in vivo. Presumably, these mutations increase the activity of the promoter by creating higher-affinity binding sites for Spo0A. This has been demonstrated by footprint analyses for the −38C mutation (19). On the basis of the DMS protection assays and mutational analysis of the *spoIIG* promoter, we conclude that site 1 contains one strong Spo0A-binding sequence whereas site 2 has two weaker binding sequences. There is a second sequence in site 1 (−84 to −78) that is somewhat similar to an 0A box (5′-TTAAGCA-3′) (Fig. 6). This region is protected from DNase by Spo0A, but if Spo0A closely contacts this sequence we would expect to find that the G at position −79 of the transcribed strand was protected in the DMS experiment, and that was not observed. Furthermore, a base-pair substitution at −78 that changes this sequence to be less similar to an 0A box actually

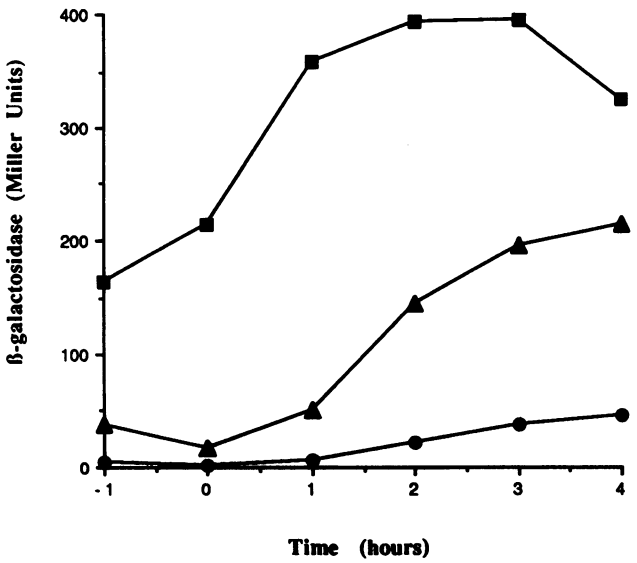

FIG. 11. The −47G change in the *spoIIG* promoter increases promoter activity in vivo. *B. subtilis* strains containing *spoIIG* promoter-*lacZ* fusions were grown in DSM media. Samples were taken from growing cultures at mid-log phase (T_{-1}), at the end of exponential growth (T_0), and at 1-h intervals after the onset of stationary phase (T_1 to T_4) and then assayed for β-galactosidase activity. All promoter mutations were assayed in *B. subtilis* BG306 (wild-type *spo0A*). Circles, wild-type *spoIIG* promoter; triangles, −47G promoter; squares, −38C/−47G promoter.

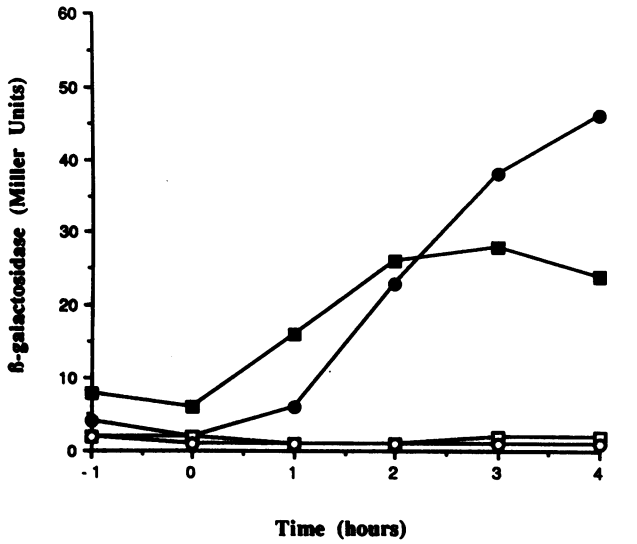

FIG. 12. The −38C/−47G *spoIIG* promoter can be activated by nonphosphorylated Spo0A in vivo. *B. subtilis* strains containing various alleles of *spo0A* and *spoIIG* promoter-*lacZ* fusions were grown in DSM media. Samples were collected in the same manner as described in the legend to Fig. 11. Open circles, BG314 (D56Q *spo0A*) and wild-type *spoIIG* promoter; open squares, BG307 (Δ*Hpa*I-*Bgl*II *spo0A*) and −38C/−47G promoter; closed circles, BG306 (wild-type *spo0A*) and wild-type *spoIIG* promoter; closed squares, BG314 and −38C/−47G promoter.

increases promoter activity in vivo (18). These results also suggest that Spo0A is the only factor that directly regulates *spoIIG* promoter activity since nearly all mutations that affect promoter activity in vivo (18; this study) are located in the 0A boxes or in putative RNA polymerase binding sites.

The *spoIIA* promoter also has multiple sequences that are similar to the 0A box and are located in the regions protected by Spo0A in DNase and DMS protection assays. However, the arrangement of sequences differs from those of the *spoIIG* promoter. The upstream region that was most affected by phosphorylated Spo0A contains two 0A box-like sequences except these are in the reverse orientation to the 0A boxes of the *spoIIG* promoter. There is also evidence that Spo0A contacts sequences adjacent to these 0A boxes, perhaps by some cooperative interactions. The downstream region of the *spoIIA* promoter contains a low-affinity binding site for Spo0A as shown by DNase I footprint assays. DMS protection assays suggest that there is a single weak Spo0A-binding site (−22 to −16) in this region. The location of this sequence and the observation that a high concentration of Spo0A is required for protection suggest that this sequence may be important for the inhibition of expression from the *spoIIA* promoter later in sporulation when Spo0A has accumulated to its highest level.

We compared the sequences for which there is experimental evidence that they signal Spo0A binding in several promoters (Fig. 14). On the basis of this comparison, we suggest that the sequence 5′-TGTCGAA-3′ appears to form an optimal Spo0A-binding site. This sequence differs from the previously reported 0A box 5′TGNCGAA-3′ (21) by a T in the third position. We believe that a T in this position is important since it is conserved in most known 0A-binding sites and previously reported mutations in the *spoIIG* promoter which change this base in the 0A boxes of site 2 (positions −36 and −46) to be less like the consensus sequence decrease promoter activity in vivo (18). The sequence 5′-TGTCGAA-3′ was sufficient to create a Spo0A-binding site in the polylinker region of pUC19. Protection of this site was increased to a small extent by phosphorylation of Spo0A. A site with a single base substitution (5′-TGTCGAG-3′) was not protected by the same concentration of Spo0A. This lower-affinity sequence is the same as the sequence found in site 1 of the *spoIIG* promoter. A mutation in the *spoIIG* promoter which makes site 1 identical to the consensus sequence increases promoter activity in vivo by approximately 20% (data not shown).

A somewhat unexpected result of this work is that phosphorylation of Spo0A increases its affinity for one site (site 2) more than for another, the upstream site (site 1). This observation could not be explained entirely by an increase in cooperative binding between sites 1 and 2, since there is an increased affinity for site 2 even when site 1 has been deleted. This observation suggests that phosphorylation of Spo0A affects its affinity for some sites more than others. One implication of this interpretation is that there may exist two classes of Spo0A-binding sites. One class may respond to changes in the concentration of nonphosphorylated Spo0A, and a second class may respond only to phosphorylated Spo0A. We note that after the onset of sporulation, Spo0A becomes relatively abundant. In Western blot analyses with anti-Spo0A antibody we compared the intensities of the Spo0A band found in the crude extracts to those produced by analysis of serial dilutions of purified Spo0A and estimated that Spo0A represents approximately 0.1% of the total soluble protein in cells 3 h after the onset of sporulation (data not shown). The fraction of this protein that becomes phosphorylated during sporulation is not known.

It is also not known why phosphorylation of Spo0A affects its affinity for some sites more than others. The sites in the *spoIIG* and *spoIIA* promoters at which affinity of Spo0A is most

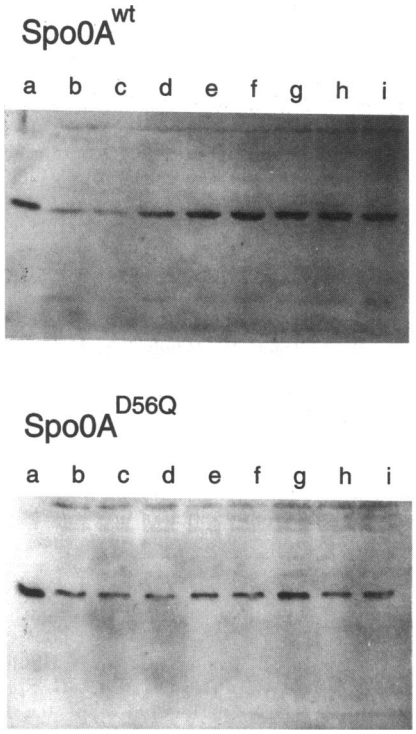

FIG. 13. Western blot analysis of Spo0A accumulation. Strains containing either the wild-type (top panel) or the D56Q (bottom panel) allele of *spo0A* were grown in DSM. Portions of the cultures harvested 1 h before the end of exponential growth phase (lanes b), at the end of exponential growth (T_0) (lanes c), and at subsequent 0.5- or 1-h intervals ($T_{0.5}$ [lanes d], T_1 [lanes e], $T_{1.5}$ [lanes f], T_2 [lanes g], T_3 [lanes h], and T_4 [lanes i]) were sonicated, and samples containing 50 μg of protein were subjected to electrophoresis in a 12% (wt/vol) polyacrylamide gel containing SDS. Purified Spo0A (50 ng) was applied in lanes A. After electrophoresis, blots of the gels were probed with rabbit anti-Spo0A antibody as described in Materials and Methods.

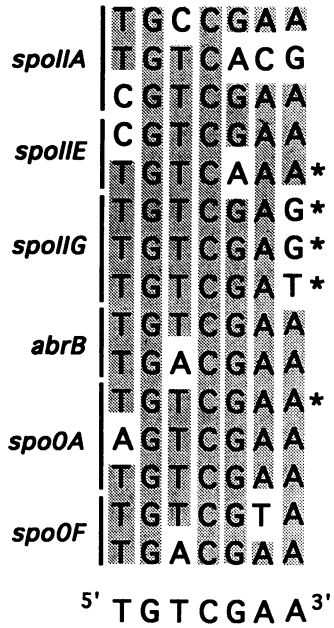

FIG. 14. Spo0A-binding site. The order of the sites are from the most upstream to the most downstream and are located in the following positions in the promoters: *spoIIA* (Fig. 6); *spoIIE*, −74 to −68 and −41 to −35; *spoIIG* (Fig. 9); *abrB*, +11 to +17 and +21 to +27; *spo0A*, −141 to −135, −99 to −93, and −11 to −5; *spo0F*, −61 to −55 and +9 to +15. The sequences shown are those that have been studied by either mutational analysis, DMS protection assays, or DNase footprint assays (*spoIIA* [this paper], *spoIIE* [28], *spoIIG* [18, 19, and this paper], *abrB* [21], *spo0A* [23], and *spo0F* [22]). For the second and third *spoIIG* binding sequences and the second *spoIIE* sequence, we replaced wild-type bases with single base substitutions that, when present, increase the promoter activity in vivo by creating better binding sites for Spo0A. The asterisks indicate sequences found in the opposite orientation, relative to the start point of transcription, from that of the sequences without the asterisks. Shading indicates bases similar to those in the consensus sequence.

affected by phosphorylation consist of weak 0A boxes. Although phosphorylation does not appear to affect cooperative binding between sites 1 and 2 of the *spoIIG* promoter, it may affect cooperative interactions between Spo0A binding at the two weak 0A boxes in site 2 and between Spo0A binding at the 0A boxes and adjacent sequences in the *spoIIA* promoter. However, phosphorylation of Spo0A also affected its affinity for the single strong site in pUC19. It appears that some features of the sequence context that surrounds each 0A box may affect the relative affinities of phosphorylated and nonphosphorylated Spo0A.

We suggest that when both Spo0A-binding sequences in site 2 of the *spoIIG* promoter are mutated toward consensus (−38C/−47G *spoIIG*), promoter activation is no longer dependent upon phosphorylation of Spo0A since it is still activated in the D56Q *spo0A* background. The mutant *spoIIG* promoter is less active in the D56Q *spo0A* strain than in the wild-type *spo0A* background, but Western blot analysis suggests that this may, in part, reflect a lower concentration of Spo0A in the D56Q strain. Since the addition of strong binding sites in the *spoIIG* promoter can partially suppress the effect of the D56Q substitution in Spo0A, we suggest that a primary role of Spo0A phosphorylation in vivo is to increase its affinity for a specific set of binding sites, rather than to activate an activity of Spo0A that acts on RNA polymerase at promoters. Some promoters possessing strong binding sites may respond primarily to an elevation in levels of Spo0A protein, regardless of its phosphorylation state.

We observed that the 0A boxes just upstream from the −35 regions in the *spoIIG* and *spoIIA* promoters have the opposite orientations. The two orientations of 0A boxes may indicate that Spo0A can contact the transcription apparatus in two different ways. It is not known whether a specific orientation of the 0A box is always associated with σ^A-dependent promoters and the other orientation is associated with σ^H-dependent promoters. It will be interesting to determine whether the interactions between Spo0A and σ^A- or σ^H-RNA polymerases are different or whether two different sets of interactions between Spo0A and RNA polymerase are possible regardless of which sigma factor is present.

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